Survival of Spacecraft-Associated Microorganisms under Simulated Martian UV Irradiation

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Spore-forming microbes recovered from spacecraft surfaces and assembly facilities were exposed to simulated Martian UV irradiation. The effects of UVA (315 to 400 nm), UVA+B (280 to 400 nm), and the full UV spectrum (200 to 400 nm) on the survival of microorganisms were studied at UV intensities expected to strike the surfaces of Mars. Microbial species isolated from the surfaces of several spacecraft, including Mars Odyssey, X-2000 (avionics), and the International Space Station, and their assembly facilities were identified using 16S rRNA gene sequencing. Forty-three *Bacillus* spore lines were screened, and 19 isolates showed resistance to UVC irradiation (200 to 280 nm) after exposure to 1,000 J m^{-2} of UVC irradiation at 254 nm using a low-pressure mercury lamp. Spores of *Bacillus* species isolated from spacecraft-associated surfaces were more resistant than a standard dosimetric strain, *Bacillus subtilis* 168. In addition, the exposure time required for UVA+B irradiation to reduce the viable spore numbers by 90% was 35-fold longer than the exposure time required for the full UV spectrum to do this, confirming that UVC is the primary biocidal bandwidth. Among the *Bacillus* species tested, spores of a *Bacillus pumilus* strain showed the greatest resistance to all three UV bandwidths, as well as the total spectrum. The resistance to simulated Mars UV irradiation was strain specific; *B. pumilus* SAFR-032 exhibited greater resistance than all other strains tested. The isolation of organisms like *B. pumilus* SAFR-032 and the greater survival of this organism (sixfold) than of the standard dosimetric strains should be considered when the sanitation capabilities of UV irradiation are determined.

When vegetative cells of *Bacillus* species are confronted with low nutrient abundance, the bacteria can initiate the process of sporulation, in which the growing cells differentiate into dormant spores (48). Spores do not metabolize at a detectable level and are highly resistant to several perturbations, such as heat and exposure to UV and gamma radiation (33). Due to this intrinsic resistance spores are ubiquitous in the environment and have been found both above and below the surface of the Earth (33, 38).

The resistance of spores has prompted agencies such as the National Aeronautics and Space Administration (NASA), the Department of Homeland Security, and others to study sporulating bacteria more closely (1, 2, 9, 11, 15, 36, 43, 50). Recently, in several microbial diversity surveys performed over a period of 3 years, 125 aerobic microbial strains were isolated from spacecraft assembly facilities (20, 24, 25, 52, 53), and their phylogenetic affiliations were determined (23, 54). Eighty-five percent of these strains were identified as gram-positive bacteria. About 65% of the strains cultivated survived heat shock protocols used to isolate sporulating bacteria (2). Members of the genus *Bacillus* were the predominant microbes among the heat shock survivors (>91%). A total of 15 different *Bacillus* species were identified. *Bacillus licheniformis* was the most

prevalent species (25%) and *Bacillus pumilus* (16%) was the second-most prevalent species isolated from spacecraft assembly facilities.

Dormant spores of various *Bacillus* and *Clostridium* species are much more resistant than their vegetative cell counterparts to a variety of treatments, including UV irradiation (49). UV irradiation has been used as a sporicide to decontaminate or sterilize surfaces, air, and water (5, 19, 40, 56). Previous work has demonstrated that binding of α/β-type small acid-soluble proteins to spore DNA is the predominant if not sole determinant of spore UV resistance (47). Further studies using *Bacillus subtilis* spores implicated several genes, including *dacB*, *spl*, and *ssp*, as genes that are essential in UV irradiation resistance (37). In addition, the spore coat has been shown to provide intrinsic protection against UVA radiation (315 to 400 nm) and UVB radiation (280 to 315 nm) (39). However, there have been few studies that have tested the UV resistance of a diverse range of environmental strains of *Bacillus* species (28). Invariably, all microbial lethality assessments and sterilizer validation studies have been carried out using laboratory strains of *B. subtilis* and/or *Geobacillus stearothermophilus* (32). Microbes inhabiting nutrient-poor and dry spacecraft assembly facilities and the resistance traits of these organisms are important to the aerospace, medical, and pharmaceutical industries. There has been growing concern that the hardy nature of spores could allow them to escape their earthly confines, survive in the vacuum of space, and intercept extraterrestrial bodies (12, 29). In addition to these concerns, the resistance of spores could enable the organisms to escape sterilization pro-

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tocols and possibly survive aboard spacecraft to contaminate planets or contaminate critical industrial implements.

In this study, we screened endospores of isolated spore-forming bacteria from a growing collection of spacecraft and associated environmental isolates for resistance to UV irradiation at 254 nm (UV_{254}). In order to examine the biocidal effects of direct UV irradiation predicted for Mars, UV_{254}-resistant spores were exposed to simulated Martian UV irradiation. The effects of UVA, UVA+B (280 to 400 nm), UVC (200 to 280 nm), and total UV irradiation (200 to 400 nm) on the survival of microorganisms at intensities expected to strike the surface of equatorial Mars were examined.

MATERIALS AND METHODS

Isolation and identification of microbes from spacecraft surfaces and associated assembly facilities. Species of *Bacillus* were previously isolated from surfaces of the Mars Odyssey and X-2000 (avionics) spacecraft and their associated assembly facilities at the Jet Propulsion Laboratory (JPL), Kennedy Space Center (KSC), and BAE Systems (Manassas, VA). *Bacillus* species were also isolated from surface samples taken in the International Space Station. Surface areas of approximately 25 cm^2 were sampled using water-moistened, sterile polyester swabs (catalog no. TX761; Texwipe). Selected floor surfaces with areas of approximately 3,600 cm^2 were sampled using sterile wipes (catalog no. TX1009; Texwipe). The swab and wipe samples were placed into sterile polypropylene bottles containing 10 and 100 ml sterile water, respectively, and transferred to the laboratory for analysis. Swabs exposed to the assembly facility atmosphere but not used in active sample collection served as controls. The spore-forming bacterial populations were selected by sonicating the samples for 2 min and heat shocking them at 80°C for 15 min. Appropriate aliquots of samples were placed into petri dishes in duplicate using the pour plate technique with Trypticase soy agar (TSA) (Becton Dickinson and Co.) as the growth medium. Following 2 days of incubation at 32°C (1, 2) isolates were selected, purified, and stored at −80°C for further processing and analysis. All other strains were purchased from the American Type Culture Collection and are listed in Table 1.

The identities of purified strains were determined by using 16S rRNA gene sequencing. Bacterial small-subunit rRNA genes were PCR amplified with eubacterially biased primers B27F and B1492R (26). PCR conditions described elsewhere were used (41). The PCR-amplified 16S rRNA gene fragments were purified using Qiaquick columns (QIAGEN) and were fully, bidirectionally sequenced. The phylogenetic relationships of organisms covered in this study were determined by comparison of individual 16S rRNA gene sequences to sequences in the public database described at http://www.ncbi.nlm.nih.gov. Evolutionary trees were constructed by phylogenetic analysis using parsimony software described at http://paup.csit.fsu.edu. The sources of the strains tested and their 16S rRNA gene sequences and GenBank accession numbers are shown in Table 1.

Sporulation. A nutrient broth sporulation medium was used for the initial sporulation of test bacteria (30, 45). A single purified colony was inoculated into liquid nutrient broth sporulation medium and incubated at 32°C, and the cultures were examined by using wet mounts and light microscopy each day to determine the level of sporulation. Once the number of free spores in each culture was greater than 99% of the total number of cells present, typically 2 to 3 days, cultures were harvested by centrifugation. Spore purification was performed by treating the spores with lysozyme and washing them with salts and detergent, as described by Nicholson and Setlow (30). Purified spores were resuspended in sterile deionized water, heat shocked at 80°C for 15 min, and stored at 4°C in glass tubes until they were used.

Selection of UV_{254}-resistant spores. Purified spores of 44 strains (Table 1) were diluted in phosphate-buffered saline (pH 7.2) to obtain a density of 10^6 spores per ml. The initial spore density was estimated by the dilution plating method before each exposure. A low-pressure handheld mercury arc UV lamp (model UVG-11; UVP, Inc.) was placed over a sample, and the UV flux at the surface of the spore suspension was measured using a UVX digital radiometer (UVP, Inc.). The exposure time required to produce 1,000 J m^{-2} of energy at the sample surface was determined to be 167 s at 600 μW cm^{-2}. The spore suspension was placed in an uncovered 100-mm glass petri dish containing a magnetic stir bar and was exposed to UV_{254} irradiation under sterile conditions. In a qualitative screening analysis, strains surviving 1,000 J m^{-2} irradiation were selected for quantitative lethal dose curve analysis. Samples (100 μl) were removed after specific periods of time, serially diluted, and plated on TSA. The

dose at which 90% of the spores were inactivated (LD_{90}) was >200 J m^{-2} were considered resistant and were used for further experiments.

Mars atmospheric radiation model. Figure 1 compares the output of a Mars atmospheric irradiation model with the spectral irradiances used in the lab experiments. The Mars atmosphere UV model used in this study was developed using Mars Pathfinder data for visible light to near-infrared light and atmospheric transmittance models developed at the University of Arizona Lunar and Planetary Lab (51). Expansion of the model into the UV bandwidth was accomplished by extrapolation and by comparing the results at specific wavelengths to observational data for Mars dust-like materials (unpublished data). Four conditions were modeled to explore the range of UV irradiance for a likely lander site (Fig. 1). Two levels of dust were modeled (optical densities at 671 nm of 0.24 and 0.74), and these levels correspond to the range of dust levels experienced by the Opportunity rover (4). The high-sun conditions were typical for a lander with the sun directly overhead, a solar zenith angle of 2.06°. The low-sun conditions simulated a position 45° south during the Martian winter, with the same two optical densities of dust. Here the solar zenith angle was 70.2°, which significantly reduced the UV irradiance due to dust absorption. The spectral shapes are very similar for the four model spectra since the shape of a spectrum is affected by dust absorption and scattering. Ozone absorption or ice scattering was not modeled as these conditions are not the most common conditions.

Resistance of spores in water to simulated Mars UV irradiation. Spores resistant to UV_{254} irradiation were exposed to UVA (315 to 400 nm), UVA+B (280 to 400 nm), or total UV (200 to 400 nm) at a simulated Mars constant level of total solar irradiance of 590 W m^{-2} (190 nm to 3 μm) (3) using an X-25 solar simulator (Spectrolab Inc.) equipped with a xenon arc lamp located at the Environmental Test Laboratory, JPL. The intensity of UV irradiation was approximately 10% of the intensity of the full spectrum (59.0 W m^{-2}). The total level of irradiance in these simulations matched the actual Mars constant level of solar irradiance, 590 W m^{-2} (3). The intensity of the irradiation was constantly adjusted by fine-tuning the lamp power to achieve ±5% variability, as indicated by an Optronics Laboratories OL754 spectroradiometer. The various bandwidths were generated by using Corning glass (UVA) or plastic petri dish lids (UVA+B; Fisher Scientific) as filters in front of the sample cuvettes. The lethal doses were calculated for each strain in order to estimate LD_{50}, LD_{90}, and LD_{100} UV dosages. LD_{100} was defined as the UV dose at which no cultivable spores were recovered using the procedures described here.

Spores of nine strains that exhibited the highest LD_{90} were diluted with sterile deionized water to obtain approximately 1×10^6 spores ml^{-1}. Then 2-ml aliquots of the suspensions were placed in 3-ml Suprasil quartz cuvettes (path length, 10 mm) equipped with a micro stir bar (3 by 3 mm; Fisher Scientific). When two strains were mixed for exposure, 5×10^5 spores ml^{-1} of each strain were mixed to obtain a final density of 10^6 spores ml^{-1}. The UV exposure times ranged from 30 s to 30 min. At various intervals, 100-μl samples were removed, diluted serially 10-fold in sterile phosphate-buffered saline, and plated onto TSA. All TSA plates were incubated at 32°C for 24 to 48 h, and CFU were enumerated. The most resistant spores were selected for further experimentation. Quartz cuvettes were cleaned after each exposure experiment by rinsing them three times with 70% ethanol and then three times with 95% ethanol. The rinsed and dried cuvettes were placed in appropriate gas-permeable envelopes (Tyvek pouches; Advanced Sterilization Products) and sterilized by exposure to one to four cycles of hydrogen peroxide injection in a Sterrad 100S vapor hydrogen peroxide sterilizer (Advanced Sterilization Products).

In addition to the X-25 system spores of two strains, *B. subtilis* 168 and *B. pumilus* SAFR-032, were exposed to simulated Martian irradiation using a xenon arc lamp (model 6262; Oriel Instruments) located at KSC under spectral conditions reported previously (46). Various bandwidths were generated as described above. In all UV assays in which we examined a mixture of *B. subtilis* 168 and *B. pumilus* SAFR-032 enumeration was possible due to the distinctly different colony morphologies of the two organisms while they were growing on agar medium.

RESULTS

Identification of UV irradiation-resistant microbes. Initially, samples from several spacecraft surfaces and associated assembly facilities were subjected to a heat shock protocol (2) to isolate sporulating bacteria. Spores of 43 strains (Table 1) were screened for UV_{254} resistance using an Hg lamp. Nineteen strains exhibited growth after receiving a dose of 1,000 J m^{-2}. Phylogenetic analyses were performed for all the strains tested,

TABLE 1. Characteristics of microbes associated with several spacecraft and associated environments tested in this study

Species	Strain[a]	Source[b]	Location[c]	GenBank accession no.	Qualitative resistance to UV_{254}[d]	Reference
B. atrophaeus	ATCC 9372	Raven Lab	NASA standard strain (formerly *B. globigii*)	X60607	−/−	38
B. benzoevorans	ATCC 49005[T]	ATCC	Soil in France	D78311	−/−	38
	SAFN-024	Clean room floor	JPL-SAF	AY167808	+/+	This study
B. cereus	ATCC 14579[T]	Air	ATCC	AF290547	+/+	38
	FO-11	Clean room air particulates	JPL-SAF	AF234842	−/−	24
B. firmus	ATCC 14575[T]	ATCC	NA	X60616	+/+	38
	10V2-2	Clean room floor	KSC-PHSF	AF526919	+/+	23
B. flexus	ES-16	Clean room table	Manassas, VA	NA	−/−	This study
B. fusiformis	ATCC 7055[T]	ATCC	NA	L14013	+/+	38
B. gibsonii	ATCC 700164[T]	ATCC	NA	X76446	−/−	38
B. licheniformis	KL-196	Clean room floor	JPL Vibration Testing Facility	AF387515	+/+	52
B. megaterium	ATCC 14581[T]	ATCC	Milk	X60629	+/+	38
	KL-197	Entrance floor	JPL-Highbay	AY030338	−/−	52
B. mojavensis	ATCC 51516[T]	ATCC	Soil, Mojave Desert, Rosamond, CA	AB021191	−/−	38
	KL-154	Entrance floor	JPL-MECA	AY030334	−/−	52
B. mycoides	ATCC 6462[T]	ATCC	Soil	X55061	+/+	38
	FO-080	Clean room air particulates	JPL-SAF	AF234860	−/−	54
B. nealsonii	BAA-519[T]	Clean room air particulates	JPL-SAF	AF234863	−/−	53
B. neidei	NRRL BD-101[T]	USDA	Soil	AF169508	+/+	23
B. niacini	51-8C	Clean room floor	KSC-PHSF	AF526905	+/+	54
B. odysseyi	PTA-4993[T]	Mars Odyssey spacecraft	KSC-PHSF	AF526913	+/+	25
B. psychrodurans	VSE1-06	Clean room air particulates	KSC-PHSF	AJ277983	+/+	This study
B. pumilus	ATCC 7061[T]	ATCC	NA	AB020208	−/+	38
	ATCC 27142	ATCC	NA	AY876287	−/−	38
	51-3C	Clean room floor	KSC-PHSF	AF526907	−/−	23
	82-2C	Clean room floor	KSC-PHSF	AF526902	+/+	23
	84-1C	Clean room floor	KSC-PHSF	AF526898	−/−	23
	84-3C	Clean room floor	KSC-PHSF	AF526896	−/−	23
	84-4C	Clean room floor	KSC-PHSF	AF526895	−/−	23
	FO-033	Clean room air particulates	JPL-SAF	AF234851	−/−	20
	FO-36b	Clean room air particulates	JPL-SAF	AF234854	+/+	20
	KL-052	Clean room cabinet top	JPL-SAF	AY030327	+/+	20
	SAFN-027	Clean room floor	JPL-SAF	AY167884	+/+	20
	SAFN-029	Clean room floor	JPL-SAF	AY167883	−/+	20
	SAFN-036	Clean room floor	JPL-SAF	AY167881	+/+	20
	SAFN-037	Clean room floor	JPL-SAF	AY177880	−/+	20
	SAFR-032	Clean room floor	JPL-SAF	AY167879	+/+	20
	015342-2 ISS	Hardware	International Space Station	AY876228	−/−	This study
B. sphaericus	ATCC 14577[T]	ATCC	NA	L14010	+/+	38
B. subtilis	ATCC 6051[T]	ATCC	NA	X60646	+/+	38
	168	Wayne Nicholson, University of Arizona	Full genome sequence	NC 000964	+/+	38
	42HS-1	Clean room floor	KSC-PHSF	AF526912	+/+	23
B. thuringiensis	ATCC 10792[T]	ATCC	Mediterranean flour moth, *Ephestia kuhniella*	X55062	−/−	38
	SAFN-003	Entrance floor	JPL-SAF	AY167823	+/+	This study

[a] T = type strain.

[b] ATCC, American Type Culture Collection; USDA, U.S. Department of Agriculture.

[c] JPL-SAF, Jet Propulsion Laboratory Spacecraft Assembly Facility (Building 179); KSC-PHSF, Kennedy Space Center Payload Hazardous Service Facility; JPL-MECA, Jet Propulsion Laboratory Mars Environmental Chamber Assembly Facility; NA, not available.

[d] Growth in liquid culture determined by optical density at 600 nm/growth on agar medium determined by colony counting. +, optical density at 600 nm of >0.4 or >30 colonies after 24 h.

and the strains were unambiguously determined to be low-G+C-content gram-positive *Firmicutes* based on 16S rRNA gene sequence analysis. The 16S rRNA gene sequences of all isolates were compared, and a bootstrap analysis (500 replicates) was performed to avoid sampling artifacts. The analyses indicated that the strains tested exhibited close phylogenic relationships with *Bacillus* species. Neighbor-joining, parsimony, and maximum-likelihood analyses were performed with this subset of bacteria using several subdomains of the 16S

rRNA genes. A maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of several *Bacillus* species is shown in Fig. 2. The branching order of this tree showed that there were two distinct clusters. The top clade consisted of *Bacillus* rRNA group 1 species, and the other clade was formed by species belonging to rRNA group 2, as well as the rRNA group 1 *Bacillus* species that produce exosporium-like structures (38).

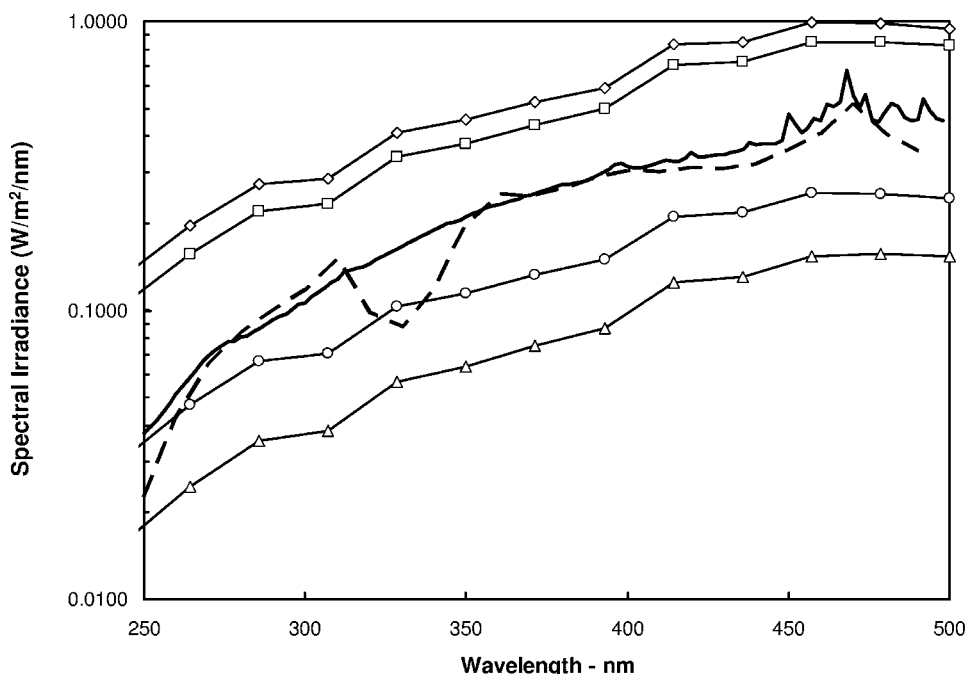The levels of 16S rRNA gene nucleotide sequence similarity

FIG. 1. Spectral irradiance plots for the JPL solar simulator (dashed line) and the KSC simulator (solid line). Also included for reference are spectral irradiance plots for the Opportunity landing site under a high sun angle and sky conditions producing optical depths of 0.24 (◇) and 0.74 (□) and irradiance plots predicted for latitude 45°S under a low sun angle and optical depths of 0.24 (○) and 0.74 (△).

between the strains tested in this study and closely related *Bacillus* species, recognized by GenBank "BLAST" searches, were between 91.6 and 99.8%. Sequence variation of <3% was observed between *B. subtilis*, *B. mojavensis*, *B. atrophaeus*, *B. licheniformis*, and *B. pumilus* (Fig. 2). The strains of *B. subtilis* and *B. pumilus* sequenced in this study were tightly bound phylogenetically, as all of them exhibited levels of 16S rRNA gene sequence similarity of >97.5%. Additionally, a high level of 16S rRNA gene sequence variation (~8%) was observed between members of rRNA group 1 and members of rRNA group 2; however, a high degree of dissimilarity within a well-described genus is not uncommon. Based on 16S rRNA analysis, five of the environmental strains tested, *B. pumilus* FO-33, FO-36b, SAFN-036, and SAFR-032 and *B. subtilis* 42HS-1, were identified as members of rRNA group 1, and two strains, *B. odysseyi* and *B. psychrodurans* VSE1-06, were identified as members of rRNA group 2 (Table 1).

Martian UV irradiation simulation. The JPL and KSC lab spectra were very similar to the models in terms of shape and level, and the KSC spectrum was more representative (Fig. 1). The JPL spectrum was deficient in 250-nm energy and exhibited a significant dip at 330 nm. This resulted in lower energies in the UVC and UVA bands and very similar levels for the UVB band. The 330-nm dip seen in the JPL spectrum was most likely due to lens coatings in the solar simulator but was not deemed to be critical because most of the absorption band was in the UVA region of the solar simulation. Our results (see below) indicated that UVA had a minimal effect on the survival of bacterial spores. The JPL and KSC simulations of Mars solar environments were calibrated to fall within a range of UV irradiation encountered under clear-sky conditions, with an

optical depth of <1, for the Mars rover Opportunity at high and low sun angles.

Survival of endospores in aqueous solution under simulated Martian UV irradiation conditions (JPL simulation). Of the 19 strains that exhibited UVC resistance, seven spacecraft-associated isolates were chosen for further study due to elevated LD_{90} of UV irradiation (data not shown) or other traits. The bacterial strains chosen and the determining factors used for selection for exposure to simulated Mars UV irradiation were as follows: *B. odysseyi* was selected because of its morphological novelty (25); *B. psychrodurans* VSE1-06 was selected because of its low-temperature tolerance; four strains of *B. pumilus* were selected because of their predominant occurrence; and *B. subtilis* 42HS-1 was selected because it is a close relative of the well-studied reference strain *B. subtilis* 168 (6, 14). In addition to the environmental strains mentioned above, *B. subtilis* 168 was selected as a control since this strain has been used in numerous other resistance studies that have been described previously (13, 14, 16, 17, 33, 46). Similarly, *B. megaterium* ATCC 14581 was also chosen for further study due to its high UV resistance compared to the UV resistance of other reference strains used in the current study. Most of the *B. pumilus* environmental isolates were obtained from the JPL spacecraft assembly facility class 100K cleanrooms; the only exception was *B. pumilus* 015342-2 ISS, which was isolated from surfaces of the International Space Station. Both *B. subtilis* 42HS-1 and *B. odysseyi* were cultured from the surface of the Mars Odyssey, and *B. psychrodurans* VSE1-06 was recovered from air samples collected in the assembly facility for the Mars Exploration Rovers, Payload Hazardous Servicing Facility, KSC (Table 1).
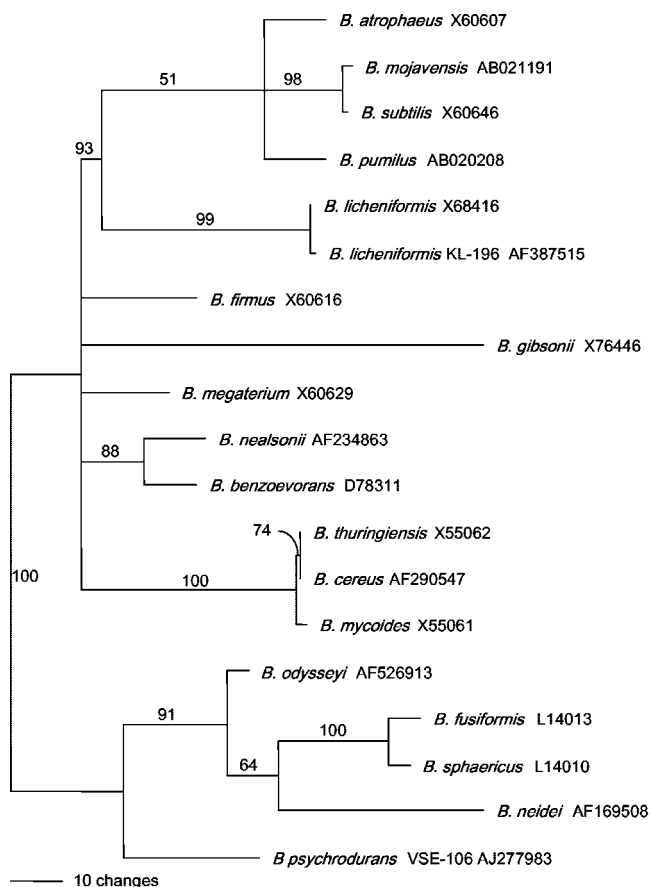
FIG. 2. Phylogenetic tree of the type strains of *Bacillus* spp. used in this study based on maximum-likelihood parsimony analysis of the 16S rRNA gene nucleotide sequences. The numbers after the names of the bacteria are GenBank nucleotide sequence accession numbers. The numbers above the lines are the bootstrap percentages for 500 replications of the branches of the tree.

The results of exposing *Bacillus* spores in aqueous solution to UVA, UVA+B, and total UV are shown in Table 2. Compared to the exposure time for the full UV spectrum, 2- to 25-fold increases in the time of exposure to UVA or UVA+B were required to reduce the viable spore counts by 50%. Like-wise, 90% reductions in viable spore numbers required 35- and 140-fold-greater times of exposure to UVA+B and UVA, respectively, than to the full UV spectrum. As shown in Table 2, none of the *Bacillus* species tested was completely eradicated even after 30 min of exposure to UVA+B irradiation or UVA (Table 2). The LD_{50}s, LD_{90}s, and LD_{100}s of the nine types of bacterial spores tested with various UV spectra in the Mars solar simulation showed that UVA+B irradiation was significantly less lethal than full-spectrum UV irradiation; therefore, as expected, the 200- to 280-nm range is more damaging than longer wavelengths. Although all spores tested exhibited sensitivity to UVA+B, most damage by UVA+B might be attributed to UVB. This was further confirmed by the observation that all the spores tested except the *B. pumilus* FO-033 spores were resistant to UVA, with growth observed even after 30 min of exposure (Table 1).

Based on the *Bacillus* species tested, resistance to UV A, UVA+B, or full-spectrum UV irradiation was found to be strain specific. However, for Mars full UV spectra, three of four *B. pumilus* strains tested in this study exhibited LD_{50}s of 40 to 80 s and LD_{90}s of 100 to 270 s. Except for *B. pumilus* SAFR-032 and *B. megaterium*, spores of the *Bacillus* species tested exhibited an LD_{50}, LD_{90}, and LD_{100} of <24 s, <60 s, and <120 s, respectively, for Mars full UV spectra. Furthermore, *B. pumilus* SAFR-032 spores that showed resistance to 2,000 J m^{-2} UV_{254} (28) were not completely killed after 30 min when they were exposed to the full UV spectrum under simulated Mars intensities. In addition, SAFR-032 spores showed greater resistance (LD_{50} of 84 s and LD_{90} of 270 s) than the spores of the other strains to exposure to the full UV spectrum under the simulated Mars solar UV irradiation conditions. Reference strain *B. subtilis* 168 exhibited LD_{50}, LD_{90}, and LD_{100} for the full UV spectrum of 24 s, 42 s, and 72 s, respectively.

Plots of the survival rates for *B. pumilus* SAFR-032, *B. megaterium*, and *B. subtilis* 168 at various times for full Mars UV irradiation are shown in Fig. 3. *B. pumilus* SAFR-032 spores exhibited classical inactivation kinetics, with a characteristic "shoulder" extending to 2 min followed by strict exponential inactivation. However, *B. subtilis* 168 and *B. megaterium* spores exhibited a sharp decline in viability immediately after 30 s of UV exposure. After 10 min of UV exposure, the cultivability of

TABLE 2. Times necessary to reduce numbers of various *Bacillus* spores that are exposed to various UV radiation conditions at the Mars equatorial solar constant

Species	Strain	Full UV (200 to 400 nm)			UVA+B (280 to 400 nm)			UVA (315 to 400 nm)		
		LD_{50}	LD_{90}	LD_{100}	LD_{50}	LD_{90}	LD_{100}	LD_{50}	LD_{90}	LD_{100}
B. pumilus	SAFR-032	1.5	4.5	>30[a]	10.0	>30	>30	8.0	>30	>30
B. megaterium	ATCC 14581	0.6	2.2	12.0	8.0	23.8	>30	7.9	>30	>30
B. pumilus	FO-036b	0.1	0.2	5.0	2.5	19.0	>30	5.0	28.0	>30
B. pumulis	SAFN-036	0.2	0.6	3.5	1.5	17.0	>30	5.1	>30	>30
B. odysseyi	34HS-1	0.3	0.8	3.5	1.8	12.8	>30	1.5	27.0	>30
B. pumulis	FO-033	0.6	1.6	10.0	1.2	2.0	>30	1.5	1.0	>20
B. subtilis	168	0.3	0.9	5.0	3.4	15.0	>30	1.5	12.6	>30
B. subtilis	42HS-1	0.4	1.4	9.5	4.0	16.2	>30	1.8	16.0	>30
B. psychrodurans	VSE1-06	0.3	0.8	5.0	6.0	23.8	>30	2.8	>30	>30

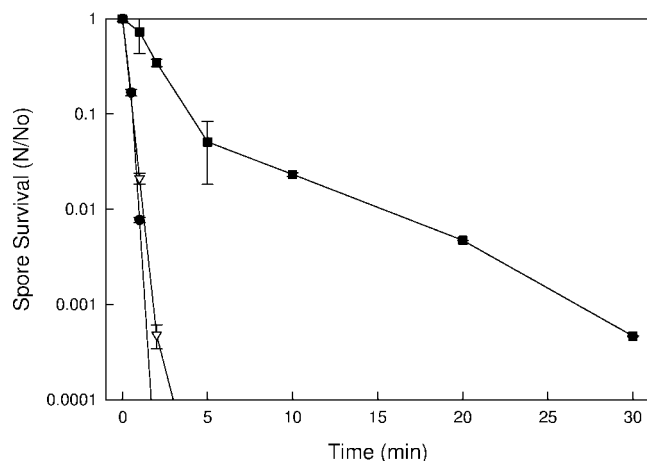[a] The maximum exposure time was 30 min.

FIG. 3. Effect of Mars UV irradiation (JPL simulator) on hydrated spores of select *Bacillus* species. ■, *B. pumilus* SAFR-032; ●, *B. megaterium* ATCC 14581; ▽, *B. subtilis* 168. The error bars indicate the standard deviations of three replicate samples. N/No, number of survivors/original number.

FIG. 5. Effect of *B. pumilus* SAFR-032 on spores susceptible to UV-induced inactivation (JPL simulator). ■, *B. subtilis* 168 in mixture; ▽, *B. subtilis* 168 alone; ●, *B. pumilus* SAFR-032 alone. In the mixed samples equal amounts of spores from the two species were mixed to obtain a total spore density of 10^6 spores ml^{-1}. The error bars indicate the standard deviations of three replicate samples. N/No, number of survivors/original number.

B. megaterium spores was completely lost, while a portion of *B. pumilus* SAFR-032 spores survived.

Effects of various UV spectra under Mars solar UV irradiation conditions on the survival of *B. pumilus* SAFR-032 spores are shown in Fig. 4. Neither UVA nor UVA+B radiation was effective in inactivating SAFR-032 spores, even after 30 min of exposure, whereas the full simulated spectrum reduced SAFR-032 spore viability by more than 3 orders of magnitude after 30 min of irradiation. When the initial concentration of spores was reduced from 10^6 spores ml^{-1} to 10^5 spores ml^{-1} and to 10^4 spores ml^{-1} before UV exposure, the spores of *B. pumilus* SAFR-032 were completely killed in 8 and 2 min, respectively (data not shown).

The effects of viable and heat-killed SAFR-032 spores on the UV-sensitive *B. subtilis* 168 spores were tested (Fig. 5). Equal portions of viable spores of *B. pumilus* SAFR-032 and *B. sub-*

tilis 168 were mixed to obtain a final density of approximately 10^6 spores ml^{-1} (5×10^5 spores ml^{-1} of each strain) and exposed to full simulated Martian UV irradiation. The time required to eradicate all *B. subtilis* 168 spores when they were mixed with an equal number of *B. pumilus* SAFR-032 spores was ~17 min, compared to 1.2 and 5 min when *B. subtilis* 168 spores at concentrations of 5×10^5 and 1×10^6 spores ml^{-1}, respectively, were exposed alone (Fig. 3 and Table 2). Heat-killed *B. pumilus* SAFR-032 spores provided no protection to *B. subtilis* 168 (data not shown).

Vegetative cells of *B. pumilus* SAFR-032, *B. subtilis* 168, and *Acinetobacter radioresistens* 50 v1, isolated from Mars Odyssey, were exposed to UV_{254} at a rate of 1 J m^{-2} s^{-1} and recovered under lighted conditions (Fig. 6). The results indicate that the three strains exhibited similar inactivation rates up to a total dose of 50 J m^{-2}, after which cells of *B. pumilus* SAFR-032 and
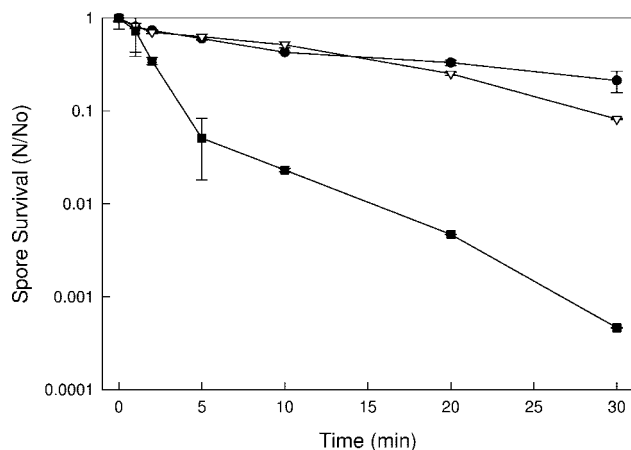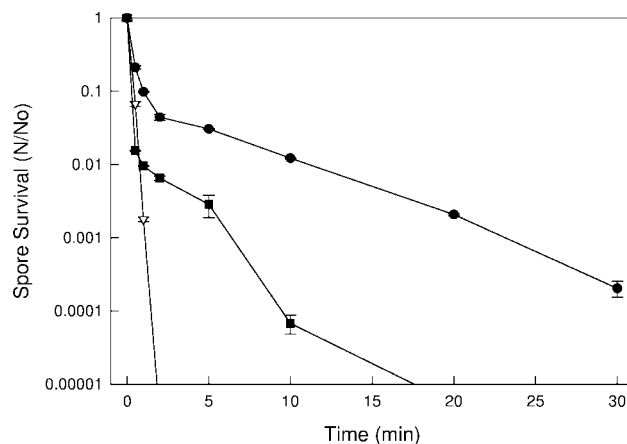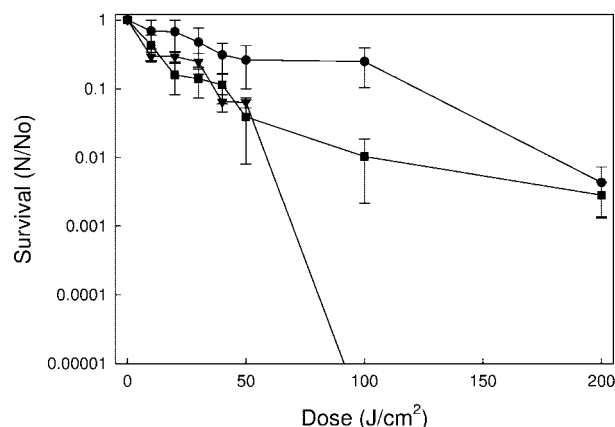

FIG. 4. Influence of various Mars UV spectra on the survival of *B. pumilus* SAFR-032 spores in water (JPL simulator). ●, UVA; ▽, UVA+B; ■, full UV spectrum. The error bars indicate the standard deviations of three replicate samples. N/No, number of survivors/original number.

FIG. 6. Effect of UV_{254} irradiation on vegetative cells. ■, *B. pumilus* SAFR-032; ●, *A. radioresistens*; ▼, *B. subtilis* 168. The error bars indicate the standard deviations of three replicate samples. N/No, number of survivors/original number.

A. radioresistens survived a 200-J m^{-2} dose (a 3-log reduction), while *B. subtilis* 168 cells were unrecoverable after a 50-J m^{-2} dose.

Survival of endospores in aqueous solutions under simulated Martian UV irradiation conditions (KSC simulation). Since several publications have shown that there are variations in Martian UV simulations (7, 21, 46), we UV irradiated spores of *B. pumilus* SAFR-032 and *B. pumilus* 168 using an alternate Martian UV simulation system developed at KSC (32). Spores of these organisms were exposed to Martian UV, UVA, UVA+B, and full-spectrum irradiation conditions. Aliquots of UV-exposed spores were plated onto TSA after UV exposure for 0, 1, 2, 5, 10, and 20 min, and the surviving spore-forming cells were counted under appropriate cultural conditions. Figure 7A, B, and C show the inactivation curves for the spores of SAFR-032 and 168 exposed to full Mars UV, UVA+B, and UVA, respectively. The LD$_{90}$ for *B. subtilis* 168 spores was <30 s when they were exposed to full Martian UV irradiation, and SAFR-032 spores exhibited at least threefold-higher resistance than 168 spores exhibited (Fig. 7A). Under the total UV spectrum the cultivability of *B. subtilis* 168 spores was lost after approximately 90 s, while *B. pumilus* SAFR-032 exhibited two- to threefold-greater resistance, remaining cultivable after 5 min (Fig. 7A). With UVA+B exposure, the survival of *B. pumilus* SAFR-032 dramatically increased, with 90% of the spores remaining cultivable, while *B. subtilis* 168 was almost noncultivable after 20 min of exposure (Fig. 7B). Similar results were observed with UVA irradiance; however, *B. subtilis* 168 remained cultivable after 20 min (Fig. 7C). It should be noted that it was not possible to tune the KSC xenon arc lamp in real time; therefore, when filters were placed in front of the light path, a 20 to 40% decrease in UV fluence occurred (57). This decrease in fluence partially explains the enhanced resistance to the two wavelengths during the experiment. However, even at the decreased fluences *B. pumilus* SAFR-032 remained more resistant than *B. subtilis* 168.

DISCUSSION

The goal of planetary protection as stated in NASA policy is prevention of forward and backward contamination (42). This policy applies directly to the control of terrestrial organisms contaminating spacecraft intended to land, orbit, fly by, or be in the vicinity of extraterrestrial bodies. Viking mission landers were terminally heat sterilized to decrease the risk of forward contamination of Mars and to ensure that terrestrial microorganisms did not contaminate the life detection experiments (42). However, the cost of designing and assembling the Viking landers was increased dramatically due to this requirement. In 1992 the Space Studies Board and the Committee on Space Research concluded from Viking mission data that Mars was less likely to support Earth-based life than previously thought (49a). The non-life-detection Mars landing missions, such as Mars Exploration Rovers, did not require all rover components to be heat sterilized prior to launch. Instead, NASA relied on a series of sequential chemical and physical sanitation steps to maintain the cleanliness of the Mars Exploration Rovers (9, 44).

The question is whether forward contamination of Mars will be significantly decreased by the inherent harsh environment at the Martian surface. Spores of *B. subtilis* have been shown to survive for up to 6 years under low-Earth-orbit conditions (15, 17). However, only shielding from UV radiation enabled *B. subtilis* endospores to survive under these conditions for a long time (15, 18). The solar flux at the Martian surface is considerably less than that experienced in interplanetary space (15, 46), and there is the potential that atmospheric conditions could further attenuate UV irradiation (7, 10).

Previous UV resistance studies have utilized model dosimetric strains and indicated that the limit for survival of organisms is approximately 200 J m^{-2} UV$_{254}$ (35). A recent study examined the survival of a laboratory strain, *B. subtilis* HA 101, on spacecraft-qualified materials under simulated Martian UV irradiance conditions (46). The results suggested that ~6 logs of spores exposed on spacecraft surfaces under the simulated UV conditions were inactivated within a few tens of minutes under Mars equatorial and clear-sky, 0.5-optical-depth conditions. Other researchers have examined a *B. pumilus* strain that was isolated from a spacecraft assembly facility and reported that it maintained one of the highest levels of UV$_{254}$ resistance reported for spores to date (28). Since most of the previously published UV resistance information has been based on the use of laboratory strains, predictions of the actual survival and possible adaptation of terrestrial life on Mars are limited due to the lack of robust empirical data. The same lack of data could also hamper efforts to use UV irradiation as a sterilization method if the most resistant organisms are not tested during the creation of dose standards. For example, the current standard for UV disinfection of drinking water is 400 J m^{-2} UV$_{254}$. *B. pumilus* SAFR-032 requires doses of 2,000 to 2,500 J m^{-2}, an order of magnitude greater than the standard, for complete sanitation (28).

The current study is the first study to report the abilities of a wide range of *Bacillus* species recovered from spacecraft and associated facilities to survive simulated Martian UV exposure. The sources of the type strains used in this study for phylogenetic comparison to spacecraft-related isolates varied from soils in France to milk (Table 1), reinforcing the widespread nature of spores on Earth. Correlations between type strain and spacecraft-related isolate resistance to UV irradiation were not observed. In some cases the type strain was more resistant to UV irradiation than spacecraft-related isolates (e.g., *B. megaterium* and *B. cereus*), but in general the spacecraft-related strains were more resistant to UV irradiation than the type strain of *B. pumilus* (Table 1). The source of spacecraft-related isolates (air or surface) did not correlate with UV irradiation resistance (Table 1).

Even though spacecraft assembly facilities are cleaned on a regular basis, it is evident that the resistance properties of spore-forming microbes allow them to adapt and persist in these environments. While it is difficult to speculate on the sources of spores within the spacecraft assembly facilities, soil is generally thought to be the main repository of spores in the environment (31) and the most likely vector of entry in this case. Evidence of this was obtained from the discovery of a strain of *B. mojavensis* in the JPL Mars Environmental Chamber Assembly Facility (Table 1). The type strain of *B. mojavensis* was isolated from a desert located within 100 miles of the JPL campus. *B. pumilus* was the predominant microbe that was repeatedly recovered from spacecraft (Viking in 1972 to Mars
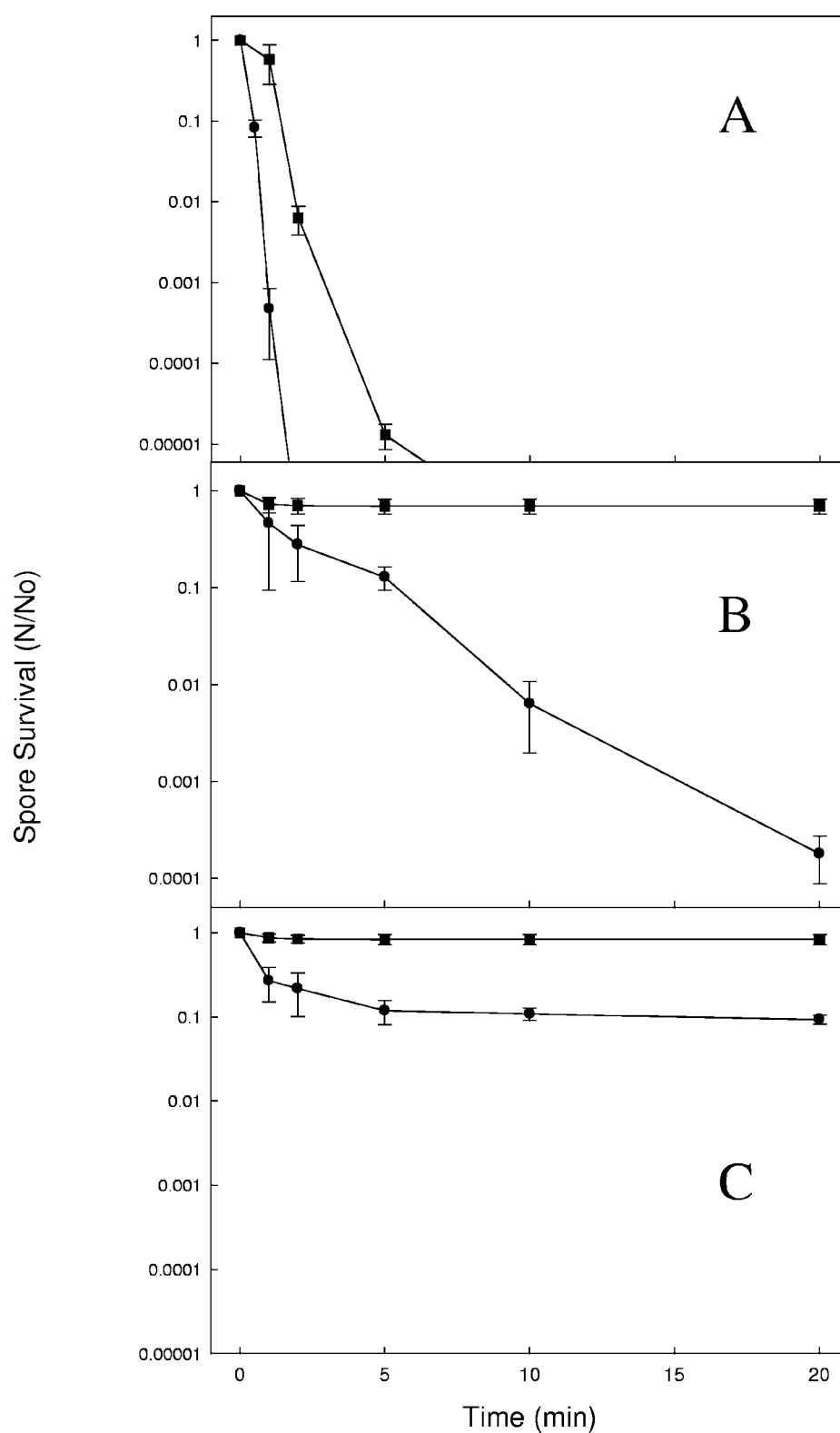
FIG. 7. Effect of Mars UV irradiation, projected from the KSC simulator, on the spores of *B. pumilus* SAFR-032 (■) and *B. subtilis* 168 (■) in water. (A) Full UV spectrum; (B) UVA+B; (C) UVA. The error bars indicate the standard deviations of three replicate samples. N/No, number of survivors/original number.

Odyssey in 2001) and the assembly facility surfaces (JPL and KSC) (22, 23, 54, 55) that resisted various perturbations, including UV and gamma radiation, and H_2O_2. There have been no reports of how this prevalent microbial species was transported into the facility or how the microbes adapted to survive in the conditions of the facility. The *B. pumilus* strains tested in this study exhibited no noticeable phenotypic differences. Recent studies employing genetic fingerprinting grouped all JPL spacecraft assembly facility *B. pumilus* isolates into three clusters (20). Therefore, it is likely that *B. pumilus* strains adapted over time to the conditions present in the spacecraft assembly facilities, and this may explain their elevated levels of resistance.

The data presented here indicate that spores of *B. pumilus* SAFR-032 are far more resistant to simulated Martian UV irradiation conditions than standard dosimetric strains are. Since *B. pumilus* SAFR-032 was isolated from a spacecraft assembly facility and exhibited enhanced UV resistance, it follows that any sanitation procedures involving UV irradiation should be based on the most UV-resistant microorganisms recovered from spacecraft. It is necessary to continue testing spacecraft contaminants in order to properly characterize the UV resistance of the viable bioload prior to launch.

Furthermore, during experiments in which spores of two different strains were mixed, it appeared that *B. pumilus* SAFR-032 spores protected the more UV-sensitive *B. subtilis* 168 spores. Specifically, colonies of *B. subtilis* 168 were not observed on plates following treatment with Martian UV irradiation for ~2 min or longer. However, when mixed with *B. pumilus* SAFR-032 spores, spores of *B. subtilis* 168 survived exposure to 5 or 10 min of Martian UV irradiation (Fig. 5). Autoclaved *B. pumilus* SAFR-032 spores did not protect *B. subtilis* 168 spores, and the inactivation curve for spores of *B. subtilis* 168 was very similar to the curves generated for unmixed 168 spores at a density of 5×10^5 spores ml^{-1}. Further research is necessary to elucidate the influence of viable *B. pumilus* SAFR-032 spores or spore components, such as spore coat proteins, on protection of UV-sensitive strains.

The spectral output of the JPL X-25 solar simulator used in this study was different than that of the KSC Martian UV simulator used by Schuerger et al. (46), as shown in Fig. 1. Many factors can contribute to the spectral quality and output of UV irradiance lamps, including the age of the lamps, special coatings on the glass bulbs, and the chemical and physical composition of the bulbs. From the UV spectral irradiance at both JPL and KSC, it is evident that the KSC UV simulation had a higher UV flux at wavelengths less than 260 nm and was more lethal to both organisms tested. UV irradiation at wavelengths less than 260 nm has been shown to be highly lethal to microbes and coincides with the action spectrum of DNA, causing the most significant damage of the UV bandwidths (18, 27, 34). Since the KSC simulator was richer in UVC than the JPL simulator, the difference in the inactivation rates supports the conclusion that UV irradiation in the 200- to 280-nm range is the UV irradiation that is most detrimental to spores. Therefore, any attenuation of UVC by dust or ice particles in the atmosphere may greatly enhance spore survival.

In summary, the results of this study suggest that the UV environment on Mars is extremely harsh and that most microorganisms exposed to the sun would be rapidly inactivated at equatorial latitudes. However, the existence of organisms like SAFR-032, whose survival was significantly greater than that of the standard lab strain, *B. subtilis* 168, should be considered when workers examine the biocidal nature of UV irradiation specifically on Mars with respect to future robotic or human exploration missions. In addition, further research is warranted (i) to determine the biocidal effects of low Martian pressure and extreme desiccation on the survival of bacteria protected from direct UV irradiation, (ii) to study the effects of Mars dust and nonbiological spacecraft residues (e.g., lubricants) on the survival of terrestrial microorganisms with Martian UV fluence rates, and (iii) to determine if low levels of diffuse UV irradiation permit adaptation of terrestrial microorganisms to different Martian conditions. The research described here demonstrated that the Mars UV environment is likely to be very detrimental to the survival of microbial species from Earth, but until the other questions mentioned above can be properly addressed, we must remain vigilant in processing spacecraft for Mars to reduce the possibility of forward contamination of landing sites.

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